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Charge-Shift Strategy for Isolation of Hemoglobin–Carcinogen Adducts Formed at the β93 Cysteine Sulfhydryl Groups

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Qualitative and quantitative analysis of human hemoglobin–carcinogen adducts has potential as a diagnostic tool for estimation of biologically effective levels of carcinogen exposure and for attaining a better understanding of individual susceptibility to chemical carcinogenesis. The purpose of this study was to devise a strategy for preanalytical enrichment of the class of covalent human hemoglobin–carcinogen adducts formed by reaction at the hemoglobin β93 cysteine sulfhydryl groups. The results define a charge-shift strategy in which a mixture composed of natural hemoglobin (Hb-SH) and low levels of hemoglobin-S–xenobiotic adducts (Hb-SX) is treated with an anionic sulfhydryl reagent (R^-), followed by anion-exchange liquid chromatographic separation of Hb-SR^- from the unreactive Hb-SX adducts. Using 4-(iodoacetamido)-salicylic acid as the charge-shift reagent, we applied the strategy to the isolation of chromatographically similar adducts with either 4-nitrosobiphenyl or [^{3}H]-*N*-ethylmaleimide. The strategy was effective for adduct concentrations ≤10 μmol/mol of hemoglobin. Application of the strategy provides an adduct-enriched fraction useful for subsequent analysis using either currently available techniques or alternate chemical or biochemical techniques that may be designed to take advantage of the enrichment procedure.

Introduction

Evidence is accumulating to indicate that analysis of covalent hemoglobin–xenobiotic adducts in animals can reflect exposure to carcinogens and their subsequent metabolic activation (*1–10*). Application of analytical methods developed for specific adducts or classes of hemoglobin–carcinogen adducts has demonstrated that exposure-related levels of adducts occur in human populations (*11–15*).

For human and animal hemoglobins, one of the principal sites of adduct formation with carcinogens and other xenobiotics is the β93 Cys sulfhydryl group, which reacts with nitrosoarenes (*4, 13, 16–19*), alkene oxides (*5, 11, 20, 21*), metabolites of halogenated hydrocarbons (*1, 3*), and metabolites of other chemicals (*10, 22–24*). For native human hemoglobin, only one sulfhydryl group (Cys β93) is available for reaction. The purpose of this study was to develop a general strategy for isolation of the family of Hb-S–xenobiotic[1] adducts present in human blood. Such a strategy would enhance efforts to identify the adducts present in human populations and would enhance the ease with which multiple types of adducts can be detected in a single sample.

Our results define a charge-shift strategy in which a mixture composed of natural Hb-SH and low levels of Hb-S–xenobiotic adducts is treated with an anionic sulfhydryl reagent, R^-. Anion-exchange liquid chromatography is then used to separate H-SR^- from the unreactive Hb-S–xenobiotic adducts.

The charge-shift strategy is illustrated with adducts formed by in vitro treatment of human hemoglobin with either [^{3}H]-*N*-ethylmaleimide (NEM) or 4-nitrosobiphenyl (4-NOB), a product of metabolism of the human carcinogen 4-aminobiphenyl (4-ABP). 4-(Iodoacetamido)salicylic acid (ISAL) was used as the charge-shift reagent.

Materials and Methods

Chemicals. For preparation of *N*-hydroxy-4-aminobiphenyl, a solution of 4-nitrobiphenyl (Aldrich; recrystallized from ethanol) in a mixture of ethanol and dimethylformamide was saturated with ammonia at 10 °C, followed by addition of H_2S (*25, 26*). The UV spectrum and extinction coefficients of the product were essentially identical with those reported previously (*27*). 4-NOB was prepared by addition of diethyl azodicarboxylate to a solution of *N*-hydroxy-4-aminobiphenyl in chloroform, followed by silica column chromatography and crystallization from ethanol (*28*). The melting point and UV absorbance spectrum were in agreement with published data (*27, 28*).

ISAL (Sigma) was crystallized from water. Aqueous solutions were prepared in 50 mM potassium phosphate; the pH was adjusted to 7.2 by addition of KOH. 4-ABP (Aldrich) was purified by addition of anhydrous HCl to a solution in benzene.

Immediately before use, a solution of *N*-[2-^{3}H]ethylmaleimide in pentane (40 Ci/mmol; New England Nuclear) was added to aqueous 0.1 M potassium phosphate (pH 7.1), and the pentane was evaporated under a stream of nitrogen.

Hemoglobin Isolation and Analysis. Washed erythrocytes from freshly drawn human blood were lysed by addition of 2 volumes of cold water. After 10–20 min, NaCl was added to 0.5 M. The clear hemolysate (about 4 mM hemoglobin) obtained by centrifugation was dialyzed overnight against 0.15 M NaCl (all at 4 °C), and portions were stored at −70 °C. All hemoglobin concentrations are expressed on a heme basis as determined by spectrophotometric measurement of cyanomethemoglobin (*29*). Hemoglobin concentrations in individual chromatographic fractions were estimated from their absorbance at 419 or 540 nm (*30*).

[1] Abbreviations: 4-ABP, 4-aminobiphenyl; 4-NOB, 4-nitrosobiphenyl; ISAL, 4-(iodoacetamido)salicylic acid; NEM, *N*-ethylmaleimide; Hb A, major form of human hemoglobin; Hb A_2 and Hb A_1, naturally occurring human hemoglobin variants; Hb-SH, any naturally occurring hemoglobin having a free β93 Cys sulfhydryl group; Hb-SAL, product of reaction of ISAL with Hb-SH; Hb-Ph_a, product of reaction of Hb-SH with 4-NOB; Hb-NES, (*N*-ethylsuccinimido)-Hb formed by reaction of NEM with Hb-SH; Hb-X, one or more unidentified hemoglobins, whose chromatographic elution volume(s) is (are) similar to that of Hb A but which (unlike Hb A) apparently do not react with ISAL.

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Reaction of ISAL with Hemoglobin. Reaction mixtures contained hemoglobin (1 mM), potassium phosphate (100 mM, pH 7.2), NaCl (50 mM), and ISAL (20 mM). Reactions at 10 °C (under CO; with stirring) were initiated by addition of freshly prepared 80 mM ISAL.

Three methods were used to monitor the extent of reaction. Most conveniently, an ion-specific electrode was used to measure the iodide concentration in a solution prepared by addition of 0.1 mL of the reaction mixture to 4.9 mL of 0.2 M sodium acetate, pH 5.0. NaI standards were prepared in the presence of hemoglobin.

For the experiment illustrated under Results, other portions (0.2 mL) of the reaction mixture were mixed with 0.04 mL of 300 mM dithiothreitol, followed by isolation of hemoglobin by gel filtration (Sephadex G-25; 20 mM Tris-HCl, pH 7.7). The spectral ratio A_{265}/A_{540} was determined for the isolated hemoglobin as a measure of hemoglobin-associated salicylyl moieties. SH concentrations in the isolated hemoglobin were measured spectrophotometrically (324 nm) at 30 °C (*21*, *31*). The reaction mixtures contained 0.1 M potassium phosphate (pH 7.15), 1 mM EDTA, 50 μM 4,4′-dithiodipyridine, and hemoglobin (≤15 μM); complete reaction required about 25 min. In the native state, each αβ hemoglobin dimer has one sulfhydryl group available for reaction, i.e., 0.50 SH per heme (*32*, *33*).

Reaction of 4-Nitrosobiphenyl with Hemoglobin. Reaction mixtures contained hemoglobin (0.5 mM), Tris-HCl (50 mM, pH 8.3), and NaCl (50 mM). While the hemoglobin solution was stirred at 30 °C under CO, portions of 11 mM 4-NOB (UV) in acetonitrile were added at 20-min intervals to incrementally increase the nominal 4-NOB concentration by 50 μM with each addition. As a control, 4-NOB was omitted from an otherwise identical reaction mixture. The progress of the reaction was monitored by adding 10 μL of 500 mM dithiothreitol to 0.5 mL of the reaction mixture (removed immediately before each addition of 4-NOB), and hemoglobin was isolated by gel filtration (50 mM Tris-HCl, pH 7.8, for kinetic measurements; 100 mM phosphate–50 mM NaCl, pH 7.2, for preparative reactions). The sulfhydryl concentrations were measured as described above.

Analysis of 4-ABP Released by Acid Hydrolysis. Hemoglobin solutions that had been treated with 1.5 N HCl for 1 h at 80 °C were made alkaline by addition of NaOH (to 2 N) and then extracted twice with hexane to recover 4-ABP released by the acid hydrolysis (*4*). The combined extracts were evaporated to dryness under a stream of N_2, and the fluorescence of a methanolic solution of the residue was measured (λ_{em} = 350 nm; λ_{ex} = 450 nm). When limited amounts of sample were available, the methanol solutions were analyzed by reversed-phase HPLC (C18 column; 65% aqueous methanol; fluorometric detection). The methods were standardized by identical treatment of mixtures of 4-ABP and untreated hemoglobin at concentrations similar to those for the samples.

Treatment of Hemoglobin with [^{3}H]NEM. A CO-saturated solution (0.25 mL) containing hemoglobin (0.1 mM), 0.1 M potassium phosphate (pH 7.1), and EDTA (1 mM) was treated with [^{3}H]NEM (0.02 mM) for 90 min at 30 °C. The hemoglobin was isolated by gel filtration, eluting with CO-saturated 10 mM potassium phosphate, pH 6.2. (Results of preliminary experiments showed that 10–20% SH modification occurred under these conditions.) The level of SH modification was determined radiometrically, the product was mixed with untreated CO–hemoglobin (in 10 mM phosphate, pH 6.2) so that the relative concentration of Hb-NES was about 1%, and the mixture was stored at −70 °C.

Ion-Exchange Chromatography. DEAE-cellulose (Whatman DE-52) anion-exchange chromatography was performed as previously described (*34*, *35*), except that a linear NaCl gradient (600 mL each of 5 and 60 mM NaCl in glycine–KCN buffer) was used, and the buffers were saturated with CO. A 1 × 25 cm column was equilibrated with glycine–KCN buffer (0.2 M glycine, 0.01% KCN, pH 7.7) at room temperature. The upper 1–2 cm of bed was composed of a 3:1 (v/v) mixture of hydrated Sephadex G-25 and DEAE-cellulose to allow more uniform adsorption of protein. After the hemoglobin (20–30 mg in glycine–KCN buffer diluted with an equal volume of water) was loaded, the column was eluted with the NaCl gradient at 35 mL/h. For comparative analyses of 4-NOB-treated hemoglobin two identical columns were eluted from a common gradient of twice the volume described above.

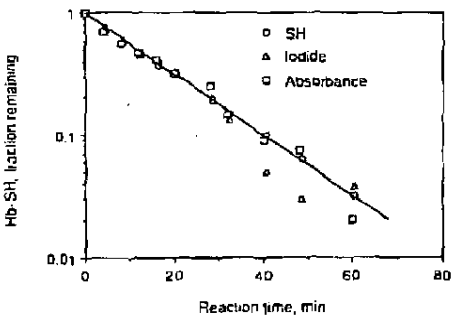

Figure 1. Reaction of ISAL with hemoglobin. The reaction mixture (10 °C) contained hemoglobin (1 mM), ISAL (20 mM), potassium phosphate (100 mM, pH 7.2), and NaCl (50 mM). At each of the indicated times a portion of the mixture was removed and mixed with excess dithiothreitol; hemoglobin was then isolated by gel filtration followed by sulfhydryl assay (SH) and measurement the A_{265}/A_{540} ratio (Absorbance). Another portion was diluted for iodide analysis (Iodide). The fractional increases in the absorbance ratio and the iodide concentration were used to compute the apparent disappearance of SH groups. The points are experimental values for duplicate analyses. The line is drawn for $t_{1/2}$ = 12 min.

Results

Reaction of Hemoglobin with ISAL. The results in Figure 1 show that the $t_{1/2}$ for the pseudo-first-order reaction of 1 mM hemoglobin with 20 mM ISAL was about 12 min, as measured by SH disappearance, iodide formation, and increased UV absorbance. Thus, the 2-h reaction period was expected to achieve >99% conversion. The amount of iodide formed (0.56 mol/mol of heme) corresponded well with the initial concentration of sulfhydryl groups (0.53 mol/mol of heme). Results of initial experiments demonstrated that, at elevated temperature and pH, additional ISAl reacted with the hemoglobin, presumably with other groups less nucleophilic than the β93 sulfhydryls.

The difference spectrum Hb-SAL vs Hb-SH provided qualitative evidence for the presence of the salicylyl moiety, although the relative absorbance values at 265 and 304 nm differed from those for unreacted ISAL. Stoichiometric incorporation of the salicylyl group increased the A_{265}/A_{540} ratio from 2.15 ± 0.01 to 2.78.

Reaction of 4-Nitrosobiphenyl with Hemoglobin. The disappearance of Hb-SH in the presence of 4-NOB is shown in Figure 2. Periodic addition of 4-NOB minimized its precipitation and also the precipitation of hemoglobin. The presence of CO minimized the 4-NOB-dependent formation of methemoglobin. In the presence of hemoglobin, the limit of solubility for 4-NOB was reached at an apparent concentration of 400 μM (uncorrected for amount reacted). The λ_{max} for the difference spectrum Hb-Ph_2 vs Hb-SH was 270 nm.

Analysis of a 60% Hb-Ph_2/Hb-SH Mixture. One 30-mg portion of 4-NOB-treated hemoglobin was treated with ISAL, while another portion was treated identically except for the absence of ISAL. The SH content of 4-NOB-treated hemoglobin, and the subsequent ISAL-dependent yield of iodide, indicated that 4-NOB reacted, respectively, with 71% or 57% of Hb-SH (Table I).

Figure 3 shows that, without ISAL treatment, Hb-SH and Hb-Ph_2 were partially resolved (evident for major and minor forms of hemoglobin), both as detected by heme absorbance (A_{419}) and the distinct A_{270}/A_{540} spectral ratios. (Figure 4 shows that only one major hemoglobin fraction

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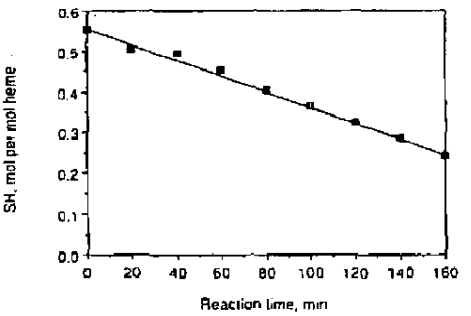

Figure 2. Reaction of 4-nitrosobiphenyl with hemoglobin. The reaction mixture contained hemoglobin (0.5 mM), Tris-HCl (50 mM, pH 8.3), and NaCl (50 mM). At each of the indicated times, 4-NOB (11 mM in acetonitrile) was added to increase its nominal concentration by 50 μM. Immediately before each addition of 4-NOB a portion of the reaction mixture was removed, and the protein sulfhydryl content was measured after isolating the hemoglobin by gel filtration.

Table I. Sequence of Reactions and Analyses[a]

reaction or procedure	analysis	SH modification, %
Hb-SH	SH; UV/vis	0; 0
↓ (+*NO-Ph*$_2$)		
[Hb-SH + Hb-Ph$_2$ + Hb-X]	SH; UV/vis	71; 65
↓ (+*ISAL*)		
[Hb-SAL + Hb-Ph$_2$ + Hb-X + I$^-$]	I$^-$	57
↓ (*chromatog*)		
separated fractions	peak areas[b]	
Hb A		61
Hb A$_2$		61

[a] The data are for the experiment illustrated in Figure 3. [b] Comparison of peak areas (A_{419}) with or without ISAL treatment.

is observed near 400 mL when the Hb-SH content is >98%.) Comparison of the two chromatograms in Figure 3 shows that conversion of Hb A to (Hb A)-SAL increased its elution volume, while the elution volume for (Hb A)-Ph$_2$ was not affected by ISAL treatment. Evidence for conversion of Hb A$_1$ to (Hb A$_1$)-SAL and Hb A$_2$ to (Hb A$_2$)-SAL is also apparent in Figure 3 and in other chromatograms presented below. These observations are consistent with the presence of β93 Cys in hemoglobins A, A$_1$, and A$_2$ (*33*). The presence and identity of Hb-X is addressed below.

The dynamic equilibrium between αβ dimers and (αβ)$_2$ tetramers is responsible for the absence of heterogeneous tetramers (e.g., αβαβ-X) in ion-chromatographic hemoglobin fractions, even when the abundance of variant hemoglobin dimers is relatively low (*36*). This characteristic of hemoglobin was confirmed by our observation that, upon treatment of the pooled (Hb A)-Ph$_2$ fraction (Figure 3, –ISAL) with HCl, 0.46 ± 0.06 (N = 5) mol of 4-ABP was released per mole of heme (0.50 expected). Furthermore, the SH contents in the pooled (Hb A)-Ph$_2$ fractions in the upper and lower chromatograms in Figure 3 were 0.021 and 0.033 mol/mol of heme, respectively (i.e., >93% SH modification either before or after ISAL treatment).

For untreated hemoglobin A_{270}/A_{540} = 2.58. A_{270}/A_{540} ratios for the most concentrated (Hb A)-Ph$_2$ fractions in Figure 3 were 3.36 (–ISAL) and 3.41 (+ISAL). Calculations based on (a) the assumption that these fractions were composed of purified (αβ-Ph$_2$)$_2$ tetramers and (b) the spectral ratio of the Hb-Ph$_2$/Hb-SH mixture before ISAL treatment indicated that 65% of the sulfhydryl groups had

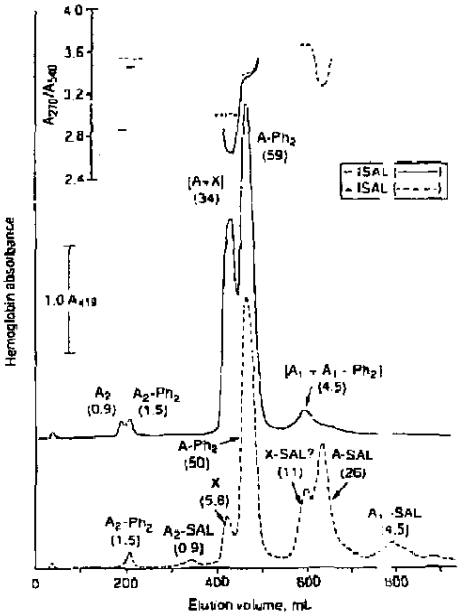

Figure 3. Ion-exchange chromatography. Two portions of 60% Hb-Ph$_2$ (prepared by treatment of hemoglobin with 4-NOB) were treated identically except for the presence or absence of ISAL. Hemoglobin from each reaction mixture was isolated by gel filtration and applied to respective DEAE-cellulose columns (–ISAL, upper; +ISAL, lower), which were eluted with a common NaCl gradient, with collection of 4-mL fractions. Values in parenthesis indicate the percent of hemoglobin (A_{419}) in the indicated fractions. The lines above the chromatogram indicate the A_{270}/A_{540} ratio (inset abscissa) as a measure of aromatic content.

been modified by 4-NOB, in good agreement with the other measures of SH modification (Table I). On the basis of the relative peak areas for spectrophotometric detection of hemoglobin (Figure 3), 61% of the β93 Cys SH groups in Hb A and Hb A$_2$ had been modified by 4-NOB (Table I).

Analysis of a 1% Hb-Ph$_2$/Hb-SH Mixture. A mixture containing 99 mol % of untreated hemoglobin and 1% (Hb A)-Ph$_2$ (obtained via the analysis shown in Figure 3) was analyzed by ion-exchange chromatography with or without prior treatment with ISAL. Comparison of the chromatograms in Figure 4 shows that ISAL treatment increased the elution volumes of Hb A$_2$, Hb A, and Hb A$_1$. The spectrophotometric peak area for the fraction designated A-Ph$_2$ was 91% of that expected from the amount of Hb-Ph$_2$ added before ISAL treatment. Furthermore, HPLC analysis of 4-ABP released by acid hydrolysis showed that, together, the regions defined by peaks X and A-Ph$_2$ accounted for at least 80% of the expected amount of 4-ABP, only 10% of which was in the region of peak X. For the most concentrated individual fractions in the Hb-Ph$_2$ region (Figure 4, +ISAL) a proportional relationship was observed between A_{419} and the yield of 4-ABP.

Treatment of Hb-NES/Hb-SH Mixtures with ISAL. Mixtures of [^{3}H]Hb-NES and untreated hemoglobin were treated with ISAL as described under Materials and Methods. Figure 5 illustrates ion-exchange chromatography of a sample in which the Hb-NES content before ISAL treatment was 150 μmol/mol of Hb-SH. Spectrophotometric detection of hemoglobin indicated the presence of (Hb A$_2$)-SAL, (Hb A)-SAL, and (Hb A$_1$)-SAL. Radiometric data showed the presence of (Hb A$_2$)-NES, (Hb A)-NES, and (Hb A$_1$)-NES having elution volumes slightly greater than the respective unmodified hemoglo-

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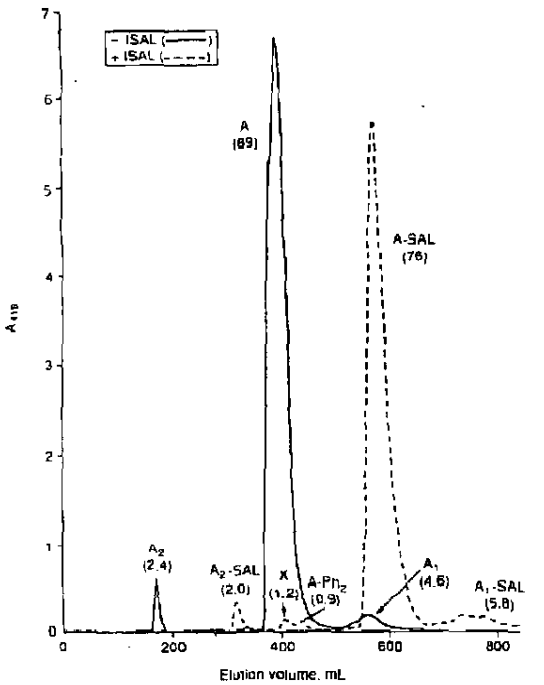

Figure 4. Ion-exchange chromatography. Two portions of 1% Hb-Ph_2 (prepared by addition of untreated hemoglobin to 4-NOB-treated hemoglobin) were treated identically except for the presence or absence of ISAL. Hemoglobin from each reaction mixture was isolated by gel filtration and applied to respective DEAE-cellulose columns (−ISAL, solid line; +ISAL, broken line), which were eluted with a common NaCl gradient with collection of 4-mL fractions. Values in parenthesis indicate the percent of hemoglobin (A_{419}) in the indicated fractions.

bins. The validity of these assignments is supported by good agreement between sepctrophotometric and radiometric measurements indicating that the amount of (Hb A_2)-SAL was 2.7% relative to (Hb A)-SAL and the amount of (Hb A_2)-NES was 2.2% relative to (Hb A)-SAL. The specific contents of the pooled fractions representing the early and late half of the radioactive Hb-NES peak were 1940 and 5520 μmol of Hb-NES/mol of heme (150 μmol/mol before chromatography). The total recovery of radioactivity was 63%, with 46% under Hb-NES.

The presence of a minor apparently ISAL-resistant Hb-X species [2.5% relative to (Hb A)-SAL in Figure 5; see also Figures 3 and 4] having the same elution volume as Hb A was unexpected in view of the kinetic data in Figure 1. On the basis of the potential explanations considered (see Discussion), we modified the procedure for isolating hemoglobin and treating it with ISAL. Specifically, freshly drawn erythrocytes were washed in CO-saturated 0.15 M NaCl/0.01 M KCN and lysed by addition of 3 volumes of 1 mM KCN. After adding NaCl to 0.5 M, the solution was centrifuged (30000g), and the supernatant was filtered (0.45 μm) and then dialyzed at 4 °C against CO-saturated 20 mM phosphate (pH 8.0) containing NaCl (0.2 M), EDTA (1 mM), and KCN (10 mM). The ISAL reaction mixture was supplemented with KCN (2 mM) and EDTA (0.1 mM), and the reaction time was increased from 2 to 3 h in the event that either Hb-SH reactivity diminishes as modification nears completion or the rate constant was somewhat less than shown in Figure 1.

A mixture of [^{3}H]Hb-NES (10 μmol/mol of heme) and hemoglobin (prepared and treated as described immediately above) was treated with ISAL (24 h after obtaining

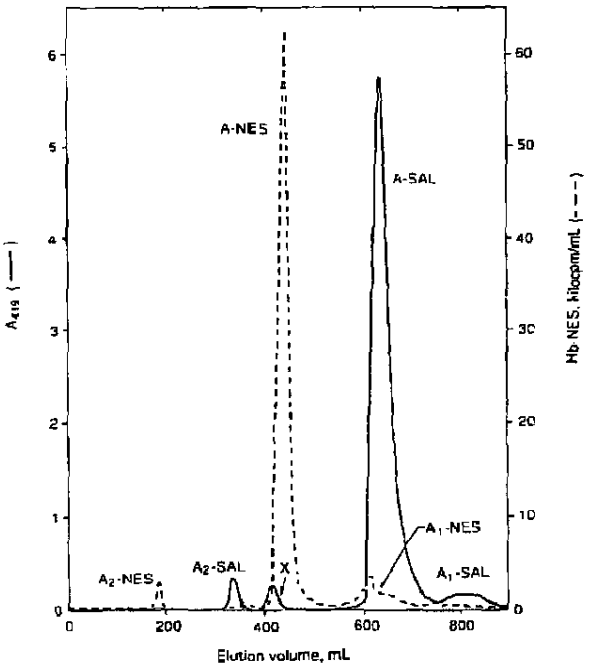

Figure 5. Ion-exchange chromatography. [^{3}H]Hb-NES (150 μmol/mol of Hb-SH) was treated with ISAL, hemoglobin was isolated by gel filtration, the hemoglobin was applied to a DEAE-cellulose column, and the column was eluted with a NaCl gradient. The radioactivity and A_{419} were measured in 4-mL fractions.

erythrocytes) and chromatographically analyzed (see Figure 6). Fractions were collected in siliconized polypropylene tubes to minimize adsorptive loss from Hb-NES-containing fractions (protein concentration ~1 μg/mL). The total recovery of hemoglobin was greater than 90% while the total recovery of radioactivity was 63% (47% under Hb-NES).[2] The relative recoveries of hemoglobin and radioactivity under the (Hb A)-NES peak indicated that the amount of Hb-NES relative to total hemoglobin was 200- to 400-fold greater than before chromatography.

The optical chromatogram in Figure 6 is distinguished from the other chromatograms presented above by the relatively low level (0.1–0.2%) of hemoglobin in the region designated Hb-X, as best illustrated by the inset showing the result of continuous monitoring (405 nm) of the effluent in the A_2-SAL region of the chromatogram. The peak area under A_2-SAL was 2.7% relative to A-SAL.

Discussion

Two principal types of analyses have been previously described for hemoglobin–carcinogen adducts that form via reaction with the β93 Cys sulfhydryl group. (a) Acid-resistant cysteine thioether adducts can be isolated by ion-exchange chromatography of acid hydrolysates and analyzed by gas chromatography or gas chromatography–mass spectrometry (37, 38). (b) Adducts (sulfinimides)

[2] The similar, relatively low recoveries of radioactivity in this and the previous experiment (see Figure 5) prompted a search for some systematic explanation. Careful validation of the radiometric methods failed to provide an explanation. Other possible explanations include (a) surface adsorption of protein from dilute solutions and (b) the presence of a radiochemical contaminant, perhaps denatured [^{3}H]Hb-NES, which was not eluted from the column.

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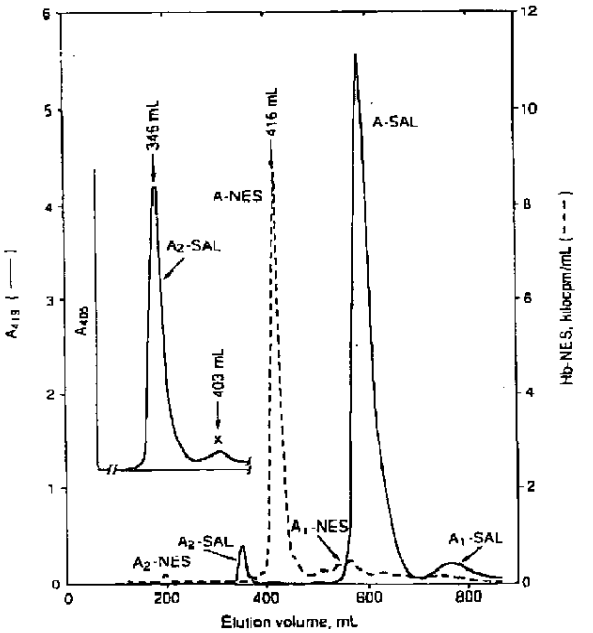

Figure 6. Anion-exchange chromatography. [^{3}H]Hb-NES (10 μmol/mol of Hb-SH) was treated with ISAL, hemoglobin was isolated by gel filtration, the hemoglobin was applied to a DEAE-cellulose column, and the column was eluted with a NaCl gradient with collection of 4-mL fractions. As described under Results, CO, KCN, and EDTA were present during the isolation of hemoglobin from erythrocytes and its treatment with ISAL. The radioactivity and A_{419} were measured in 4-mL fractions. The inset illustrates a part of the chromatogram obtained by continuous monitoring of A_{405}.

with nitrosoarenes arising from arylamine metabolism can be hydrolyzed under mild acidic or alkaline conditions, yielding the corresponding arylamines, which can be extracted, derivatized, and analyzed by gas chromatography–mass spectrometry (*4*, *13*, *39*). The second method has been used to detect exposure-related levels of hemoglobin-S-carcinogen adducts in humans (*13*, *14*, *39*).

The present results define a charge-shift strategy that provides chromatographic fractions enriched in the class of human hemoglobin–xenobiotic adducts defined by their formation at the β93 Cys SH group. The strategy relies on the reaction of hemoglobin β93 cysteine sulfhydryl groups with ISAL, an anionic derivative of iodoacetamide, the latter of which is widely used for alkylation of protein sulfhydryls. The corresponding sulfhydryl groups in thio ether and sulfimimide (*4*) adducts are not reactive with electrophilic reagents such as iodoacetamide and its derivatives. The strategy is generally applicable because several types of electrophilic mutagens and carcinogen metabolites react with hemoglobin β93 Cys SH groups, including nitrosoarenes (*4*, *13*, *16–19*), alkyl oxides (*5*, *11*, *20*, *21*, and metabolites of halogenated hydrocarbons (*3*). As determined for animal hemoglobins, metabolites of some drugs also react with the β93 SH group (*10*, *18*). We have demonstrated the effectiveness of the charge-shift strategy for isolating (a) a hemoglobin–carcinogen adduct when present at relatively high experimental levels (1% and 60%) and (b) a model adduct present at levels (10 and 150 μmol/mol of heme) approaching those that may be present in human populations. The lowest level of adduct used in this initial study was 10 μmol/mol of hemoglobin, which is 1–10^2 times greater than levels in human blood (*11–13*, *14*, *39*, *40*). However, the present results do not indicate that preanalytical enrichment of adducts present at physiologic concentrations will be problematic. The chromatographic similarity of Hb-Ph_2 and Hb-NES suggests that the procedure will allow coenrichment of several types of adducts that may be present.

Although the chromatographic data in Figures 3–5 validated the charge-shift strategy, the presence of Hb-X, an apparently ISAL-resistant minor hemoglobin, could detract from application of the procedure. Possible explanations for the presence of Hb-X included (a) the presence of an ISAL-resistant mixed disulfide, e.g., with glutathione (*41*, *42*), which yielded Hb-SH or Hb-SCN upon posttreatment exposure to cyanide (*43*) in the chromatographic buffer, (b) the presence of hemoglobin in a quaternary state in which the reactivity of the β93 Cys SH group is relatively small (*33*, *44*), e.g., deoxyhemoglobin, 2,3-diphosphoglycerate complexes with deoxyhemoglobin or methemoglobin (*45*), or certain hemichromes (*46–48*), (c) the presence of one or more hemoglobins containing chemically blocked β93 cysteine, and (d) the presence of residual unreacted Hb A. The presence of endogenous disulfide (explanation a) as the primary source of Hb-X was not supported by results of HPLC analysis of globins (as described in ref 48) before and after treatment of hemoglobin with either dithiothreitol, oxidized glutathione, or cyanide (Haugen, unpublished results).

To address the other explanations, we washed, lysed, and dialyzed erythrocytes in the presence of CO (to inhibit formation of methemoglobin and subsequent formation of hemichromes) and cyanide [to stabilize (*45*) the small amount of endogenous methemoglobin, <0.15%, and to cleave any endogenous glutathione disulfide]. The stroma-free lysate was dialyzed against 0.2 M NaCl at pH 8.0 to assure removal of all organic phosphates (*33*, *50*) that might stabilize hemoglobin in the T state for which the β93 Cys SH group is relatively unreactive. CO and EDTA inhibited oxidation of heme and sulfhydryl groups, respectively. The presence of cyanide during ISAL treatment is expected to enhance the reactivity of β93 Cys in any methemoglobin that was present (*45*). The increased reaction time better assured >99% reaction.

The results of Figure 6 show that, under the modified conditions of analysis, the amount of hemoglobin in the Hb-X region was diminished from >2% to <0.2%. The successful diminution of the Hb-X concentration by the procedures described suggests that Hb-X may be hemichrome type P (*46–48*), a partially denatured ferrihemoglobin in which the β93 Cys sulfhydryl is the sixth heme ligand, presumably inhibiting the reaction with ISAL; see explanation b. The following observations are consistent with this explanation. (i) The greatest abundance of Hb-X was in the sample treated with 4-NOBP (Figure 3), which undoubtedly resulted in partial conversion to ferrihemoglobin (*19*). (ii) The apparent sulfhydryl content of Hb-X (Figure 3) was <0.02 mol/mol of heme. Obviously, the latter observation is also consistent with the presence of hemoglobin with chemically blocked β93 cysteine (explanation c). If present, such adducts could be resolved and detected in subsequent analytical procedures.

The present results were obtained by conventional liquid chromatography of about 25 mg of hemoglobin. Modification of the chromatographic technique is anticipated to allow more timely analysis of larger samples, e.g., 100–500 mg. By increasing the sample size, sufficient adduct-enriched material can be obtained for further enrichment and for analysis using either currently available techniques or alternate chemical or biochemical techniques that may be

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designed to take advantage of the enrichment strategy. The present results show that Hb-Ph_2 and Hb-NES have essentially identical elution volumes. Further work will be required to determine whether other Hb-SX adducts have similar chromatographic characteristics.

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